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Critical considerations for fast and accurate regiospecific analysis of triacylglycerols using quantitative ^{13}C NMR

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Quantitative ^{13}C NMR (qCNMR) has been used as an appealing methodology for regiospecific analysis of triacylglycerols in edible oils and fats. It has advantages of shorter analysis time, precision and accuracy over laborious conventional Grignard or enzymatic hydrolysis method. Previous reported studies have recommended diversified NMR acquisition and processing parameters for the same quantification work. Different quantitative data were obtained by using a distinct sets of NMR parameters. To overcome this problem, we conducted a systematic investigation to examine the role of each acquisition and processing parameters to obtain high accuracy and repeatability data. Principal acquisition parameters, for instance pulse flip angle, repetition delay and temperature were investigated to correlate the targeted accuracies and practical experimental conditions. New data on spin-lattice relaxation times (T_1) for carbonyl carbons in a variety of oils and fats had been obtained and analyzed comprehensively. With this set of acquisition parameters and free induction decay (FID) data processing method, error of less than 2.0 mol% were obtained with high repeatability and versatility for the analysis of oils and fats from diverse sources, including the reaction intermediates by chemical interesterification, lipids content extracted from biological samples and those natural occurring oils without their regiospecific data reported up-to-date. Instead of the semi-quantitative approach in previous reports on fish oil, we used narrower spectral width targeting *sn*-position in triacylglycerols to obtain full quantitative data in a shorter analysis time. The present selection of data acquisition and processing parameters led to a blueprint for a generic approach to performing a routinely practiced qCNMR regiospecific analysis.

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1 Introduction

It is well-known that the types of fatty acids reside at *sn*-1, 3 (*sn* denotes stereospecific numbering) and *sn*-2 positions of triacylglycerols can cause different acute and chronic effects on atherogenicity, blood lipids, lipoprotein profile and subsequent implications for the risk of cardiovascular diseases.[1–4] Consequently, accurate and precise regiospecific analysis is of great importance. The conventional enzymatic and chemical methods have reported shortcomings, namely, problems of acyl migration by using Grignard degradation,[5] resistance of long chain polyunsaturated fatty acids towards certain lipase catalyzed analysis,[6] loss or contamination of sample during preparation,[7] and time consuming for routine analysis. For these reasons, quantitative ^{13}C NMR (qCNMR) has been used in recent studies as an appealing method to determine the positional distribution of fatty acids in the glycerol backbone of triacylglycerols in various oils and fats.

The versatile nature of NMR spectroscopy makes qCNMR an appealing method in chemical and biochemical applications in the field of natural products,[8] quantification of metabolites,[9] material science,[10,11] and analysis of lipids.[12,13] Specifically for qCNMR regiospecific analysis, carbonyl carbons in triacylglycerols are the main carbon nuclei of interest. Due to the absence of directly attached proton, carbonyl carbons have longer spin-lattice relaxation times (T_1) than other types of carbons (methyl, methylene and methine carbons). In addition to the low natural abundance of carbon-13, these pose a substantial challenge to achieve optimum signal-to-noise ratio (S/N) in a reasonable acquisition time for accurate and precise regiospecific analysis.

There is no clear guideline on data acquisition and processing parameters that can be drawn from previous reports as several assumptions have been employed on qCNMR regiospecific analyses.[14–26] In an effort to achieve shorter experimental time, some researchers suggested that the spectra could be acquired under full Nuclear Overhauser Enhancement (NOE), with the assumption that all the carbonyl carbon resonances were affected by proton decoupling to the same extent.[14,15] As a result, several published works thereafter employed broadband decoupling pulse sequence in qCNMR

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regiospecific analysis.[16–20] Yet, a recent study reported that the NOE could be different for carbonyl carbons at *sn*-1, 3 and *sn*-2 positions due to a different number of protons within five angstroms from the carbonyl carbon.[21] On the other hand, a considerable number of parameters, namely, pulse angle, pulse delay, temperature and also processing of NMR analysis differed vastly for the same regiospecific analysis work. This has resulted in diverse total experimental time per analysis ranging from the fastest 58.5 minutes[21] to 206 minutes.[22] Due to the lengthy experimental time, a semi quantitative approach was chosen in the analysis of fish oils.[17,23,24] Apart from the carbonyl carbons, other carbon atoms at *sn*-1, 3 and *sn*-2 positions of triacylglycerol show different chemical shift, and it has been confirmed through a series of INADEQUATE experiments.[25] In addition, rather complicated HSQC-TOCSY NMR spectra have been applied to analyze positional distribution of palmitoyl and oleoyl acyl chains in synthetic mixtures of pure triacylglycerols, instead of natural occurring oils and fats.[26]

Moreover, instead of 90° pulse, Ernst angle[27] and flip angles smaller than 90° [16] have been introduced in quantitative solution state NMR analysis. These have been claimed to shorten the total experimental time by decreasing the repetition time needed to allow the magnetization to recover fully before applying a new pulse. Notwithstanding the reduced repetition time, a pulse angle smaller than 90° will yield an attenuated *S*/*N*, which needs a higher number of transients for the compensation. This compromise warrants the investigation of practical and optimum pulse angle for the rapid regiospecific analysis using qCNMR.

Recently, there are several reports[27,28] detailing the protocol of quantitative ^{1}H NMR (qHNMR) on parameter-by-parameter basis in the field of natural product, yet a similar report is not available for qCNMR. In this study, we investigated the critical considerations in qCNMR acquisition and processing parameters and eventually establish a rapid, yet unbiased regiospecific analysis of triacylglycerols in diverse types of oils and fats, without subjecting the quantitative data to indiscernible errors.

2 Experimental

2.1 Reagents and materials

Standard triacylglycerols (purity ≥ 99%), namely, tripalmitoylglycerol [$C_{51}H_{98}O_6$; 1,2,3-trihexadecanoyl-*sn*-glycerol], trioleoylglycerol [$C_{57}H_{104}O_6$; 1,2,3-tri(*cis*-9-octadecenoyl)glycerol] and trilinoleoylglycerol [$C_{57}H_{98}O_6$; 1,2,3-tri-(*cis*,*cis*-9,12-octadecadienoyl)glycerol] were purchased from Sigma Chemical Company (St Louis, MO). Refined, bleached and deodorized palm olein (iodine value, IV 56), sal stearin, shea butter, cocoa butter and mango olein were obtained from Intercontinental Specialty Fats Sdn. Bhd., Dengkil, Selangor, Malaysia. Refined soybean oil, rapeseed oil (canola), grapeseed oil, rice bran oil, sunflower oil and olive oil were commercial samples purchased from local supermarkets. Menhaden fish oil was product purchased from Sigma Chemical Company (St Louis, MO). Mango fat and *Jatropha* oil were extracted using soxhlet extraction. Chemical interesterified palm olein intermediates were sampled during the reaction between palm olein IV 56 and sodium methoxide in a solvent-free system at time of 8 and 30 minutes. Lipid extract from visceral fat of C57BL/6 mice fed with palm olein and soybean oil were obtained according to approved protocol (ethic no: KIM/23/03/2011/CSF(R)) by the Animal Care and Use Committee, University of Malaya, Kuala Lumpur, Malaysia. Deuterated chloroform, $CDCl_3$, (minimum 99.8% purity) was purchased from Merck (Darmstadt, Germany).

2.2 Sample preparation

A synthetic mixture of standard triacylglycerols was prepared by mixing 99.9 mg of tripalmitoylglycerol, 248.8 mg of trioleoylglycerol and 52.0 mg of trilinoleoylglycerol and diluted with 1.0 mL of $CDCl_3$ to produce a 2 : 5 (w/v) sample–solvent ratio. The molar percentages of tripalmitoylglycerol, trioleoylglycerol, trilinoleoylglycerol were 26.7, 60.6, and 12.7 mol %, respectively. Samples of naturally occurring oils were preheated at 60 °C for 10 minutes to ensure homogeneity and then 0.2 g of the oil was dissolved in 0.5 mL of $CDCl_3$. All samples were degassed for 5 minutes by purging with inert nitrogen gas and subsequently ultrasonicated for 1 minute. The volume of sample used in NMR analysis was set at 0.5 mL for achieving the best resolution.

2.3 ^{13}C NMR spectroscopy

All ^{13}C spectra were recorded at a spectrometer frequency of 100.40 MHz fitted with a 5 mm i.d. dual probe. A spectral width of 1500 Hz in the region of acyl chain carbonyl carbon resonances, 8192 data points and 5.4 s acquisition times were employed. Due to adequate acquisition times, no linear prediction was applied. Manual phasing and baseline correction were applied to the spectrum prior to integration process. Deconvolution was done by a coefficient mixture ratio of 1 : 1 between Lorentzian and Gaussian functions and further optimized by a nonlinear least-square procedure, as described previously.[21] All spectra were acquired with 128 scans. Standard JEOL ALICE processing software was used. These influencing parameters and their justification have been summarized in Table 1.

2.3.1 Data acquisition parameters

2.3.1.1 Pulse sequence selection. Both broadband and inverse gated heterodecoupling pulse sequences were employed in the comparison. ^{13}C NMR measurements were performed on standard triacylglycerols, palm olein, rapeseed oil (canola) and soybean oil using JEOL LA-400 MHz spectrometer operating at 9.4 T, whereas JEOL ECA-400 MHz was utilized for measurement of soybean oil sample only. NOE factors $(1 + \eta)$ were calculated as the ratio of peak intensities obtained under full NOE to the NOE suppressed.

2.3.1.2 Repetition time. ^{13}C spin-lattice relaxation times (T_1) were measured using inversion recovery pulse sequence, by changing the τ values from 60.0 s to 0.1 s. The test oils were standard triacylglycerols, sunflower oil, rapeseed oil (canola), rice bran oil, soybean oil, grapeseed oil, palm olein IV 56, mango fat, olive oil and *Jatropha* oil.

Table 1 qCNMR acquisition and processing parameters used in the regiospecific analysis of triacylglycerols

Parameter	Suggested value	Justification
Data acquisition		
Data points	8192	Sufficient to define each resonance accurately (at 1500 Hz spectral width). Excessive number of data points is avoided as it will result in low *S*/*N*
Spectral width	1500 Hz, at where the carbonyl carbons resonate	Narrower spectral width at which the carbonyl carbon resonances are located near the offset to ensure the better resolution. It must cover at least an extra 5 ppm from carbonyl carbon resonances so they will fall within 80% of the central part of filter bandwidth to avoid signal attenuation
Acquisition time	5.4 s	Short yet adequate acquisition time is required. Excessively long acquisition will result in sample heating due to decoupling and collection of noise for a longer period, consequently decrement of the *S*/*N*. Acquisition time = data points/spectral width
Number of scans	128 scans + 4 dummy scans	Optimized at 2 : 5 (w/v) oil sample/solvent concentration. It is also dependent on hardware and desired *S*/*N*
Data processing		
Linear prediction	No	FID is allowed to be fully decayed within adequate acquisition time
Manual phasing	Yes	Manual phasing is favorable over automatic to ensure no distortion on the integrals of carbonyl carbon resonances
Baseline correction	Yes	To assure the quantitative accuracy and reproducibility
Smoothing algorithm	No	Artificial cosmetic valued data processing will alter original quantitative information of FID
Integration method	Deconvolution	To quantify partially overlapping signals, especially for mono- and polyunsaturated carbonyl carbon resonances, by determining the areas of individual waveforms contained within the composite profile

2.3.1.3 Pulse angle selection. ^{13}C NMR spectra were recorded using 30° and 90° pulse width. Rapeseed oil (canola) was selected as the test oil.

2.3.1.4 Temperature. The analyses were performed on rapeseed oil (canola) at the experimental temperature of 20 °C, 30 °C, 40 °C and 50 °C. The *cis*-9 monounsaturated carbonyl carbon resonance at *sn*-1,3 positions was selected for the comparison.

2.3.2 Data processing parameters

2.3.2.1 Zero filling. ^{13}C NMR spectrum of synthetic mixture of standard triacylglycerols acquired under optimum acquisition parameters determined was used to establish the set of post-processing parameters. Prior to Fourier transformation, the Free induction decay (FID) was processed under the following conditions, (I) no zero filling, (II) one level of zero filling. Accuracy of the quantitative results was examined by comparing with known composition of standard triacylglycerols mixture in five replications.

2.3.2.2 Apodization. Free induction decay (FID) was processed in exponential window. The broadening factors were fixed at 0.01 Hz, 0.1 Hz, 0.2 Hz and 0.4 Hz prior to the Fourier transformation. This investigation was done in five replications.

2.3.3 Validation on oils and fats from different sources. qCNMR with established parameters was used as the protocol for regiospecific analysis of *Jatropha* oil, shea butter, cocoa butter, mango olein, sal stearin, palm olein IV56, menhaden fish oil, chemical interesterified palm olein intermediates and visceral fat from C57BL/6 mice fed with palm olein and soybean oil for 15 weeks.

2.4 Statistical analyses

Results were expressed as mean ± standard deviation. The spectroscopic data related to NOE factors were analyzed statistically with one-way analysis of variance (ANOVA). T_1 was subjected to two-way repeated-measures ANOVA with oil type and positional distribution of acyl chains as between-subject factors. *P* values of ≤ 0.05 were considered significant.

3 Results and discussion

The assignments of carbonyl carbon resonances according to their different level of unsaturation and positional distribution within glycerol moiety are inconsistent with prior reports.[14,21,22] The group of peaks at higher frequency pertains to acyl groups from the *sn*-1,3 positions of triacylglycerol, whilst those residing at *sn*-2 position appear at lower chemical shift. Within each region, as the number of double bonds increases, the resonances appear to a higher field compared with the saturated species at the similar position. The similar trend is also observed if unsaturation occurs closer to the carbonyl carbon. Chemical shifts for acyl chains at *sn*-1,3 are shifted consistently by 0.39–0.40 ppm at higher frequencies than those of the corresponding fatty acids attached at *sn*-2, by virtue of their different γ-gauche interactions associated with steric effects.[14,21,22]

3.1 Data acquisition parameters

3.1.1 Pulse sequence selection. In an effort to obtain higher *S*/*N* ratio in a shorter experiment time, previous studies

suggested that the proton decoupled spectra of carbonyl carbons could be measured under full NOE, with the assumption that the proton decoupling affected the carbonyl carbon intensities to the same extent.[14,15] Nevertheless, the reported NOE factors[14] for *sn*-1,3 positions were in the range of 1.73–1.78, whereas at *sn*-2 position was in the range of 1.67–1.77. Statistical analysis shows that the peak intensities at both positions brought by NOE are significantly different ($P = 0.05$). Our quick calculation shows that the intensity brought by such enhancements for both positions can differ up to 6%. Due to the slightly different chemical environment and number of protons in the closest vicinity of carbonyl carbons at *sn*-1,3 and *sn*-2 positions, the heteronuclear NOE experienced by the carbonyl carbons are expected to be different for both positions during proton irradiation.[21] The different extent of the proposed enhancement will result in bias quantitative data. This leads us to investigate the effect of NOE in the present study.

Table 2 shows the NOE factors for four samples, namely, standard mixtures of triacylglycerols, palm olein, rapeseed oil (canola) and soybean oil. The NOE factors for carbonyl carbons at *sn*-1,3 and *sn*-2 positions differ significantly ($P < 0.007$), whereas there is no significant difference found for diverse degrees of unsaturation of acyl chain, oil source and also spectrometer at the same magnetic field ($P > 0.05$). Moreover, the NOE differs significantly ($P < 0.04$) for different oils in conjunction with the distribution of fatty acids at different *sn*-positions (Table 2). The NOE has very different behavior depending on the molecular motion and tumbling rate, since the enhancement has non-linear distance dependency. Therefore, temperature, magnetic field strength, solvent viscosity and concentration are the factors affecting the NOE throughout the experiment. Consequently, the actual NOE factors vary in different experiments and are subject to the condition of instrument or sample used, especially when the experimental temperature is not well controlled. This would subject the analytical results to indiscernible errors, subsequently making comparison of results obtained by different laboratories and with other instruments difficult. Based on the aforementioned reasons, the quenching of NOE in qCNMR is crucial to achieve rapid, yet unbiased quantitative analysis of positional fatty acids in triacylglycerol. The inverse gated decoupling pulse sequence has an additional advantage of minimizing sample heating and thus, suppressing the broadening effect to avoid loss of peak resolution, since the absence of proton irradiation during pulse delay will allow the thermal equilibrium to be re-established before the next acquisition.

3.1.2 Repetition time. Repetition time is the total period of the acquisition and pulse delay prior to the application of successive pulse. In order to achieve peak area that is proportional to the number of corresponding nuclei, all longitudinal magnetization, M_z, has to be returned to equilibrium magnetization, M_o, within the repetition time. The relationship among M_z, M_o, spin-lattice relaxation time T_1, repetition time, t, and pulse flip angle, θ, is depicted in eqn (1).

$$\frac{M_z}{M_o} = \frac{1 - \exp^{-\frac{t}{T_1}}}{1 - \exp^{-\frac{t}{T_1}} \cos\theta} \quad (1)$$

An optimum t can be established by having the T_1 predetermined through the inversion recovery pulse sequence. Experimental data of T_1 for carbonyl carbons in standard triacylglycerols and various types of oils are shown in Table 3. Carbonyl carbons at *sn*-1,3 positions possess significantly longer T_1 ($P < 0.01$) as compared to that at the *sn*-2 position, by virtue of the higher mobility of carbon nuclei at *sn*-1,3 positions. There is no significant difference for T_1 of carbonyl carbons at the same stereospecific numbering ($P = 0.07$). The insignificant difference is also true even for different types of oils ($P = 0.10$) (Table 3). In general, carbonyl carbon has longer T_1 due to the absence of directly attached protons, and thus poses difficulties in rapid quantitative analysis. The T_1 of carbonyl carbons reported in a previous study[14] were slightly higher than T_1 measured here, since a higher magnetic field strength is employed in the present study. The chemical shift anisotropy, which is field-dependent, is the major mechanism of spin lattice relaxation of carbonyl carbons. It is expected that at higher field, T_1 of the carbonyl carbons are shorter, therefore the experiment can be repeated more rapidly.

With the aid of eqn (1), a correlation graph between the ratio of M_z/M_o and repetition time, t, is plotted to illustrate the minimum repetition time required for achieving equilibrium at a given accuracy (Fig. 1). Our previous study reported the repetition time as 27.4 s in order to achieve 99.9% recovery of *z*-magnetization, while the longest T_1 was assumed to be 4 s.[21] Yet after a more extensive investigation on a wider range of oil samples, the actual longest T_1 for carbonyl carbons among the

Table 2 NOE ($1 + \eta$) factor for carbonyl carbons of triacylglycerols in selected oil sources[a]

	sn-1,3[d]			*sn*-2[d]		
Oil source	Saturated	Monounsaturated	Polyunsaturated	Saturated	Monounsaturated	Polyunsaturated
Standard triacylglycerols[b]	1.16	1.35	1.28	1.35	1.36	1.17
Palm olein[b]	1.36	1.33	1.11	1.50	1.29	1.24
Rapeseed oil (canola)[b]	1.30	1.37	1.26	na	1.13	1.05
Soybean oil[b]	1.27	1.56	1.39	na	1.41	1.33
Soybean oil[c]	1.18	1.37	1.27	na	1.01	1.05

[a] na = not applicable. [b] Measurements were done by JEOL LA-400 MHz spectrometer. [c] Measurement was done by JEOL ECA-400 MHz spectrometer. [d] NOE factors are significantly differed between *sn*-1,3 and *sn*-2 positions ($P < 0.007$).

Table 3 T_1 relaxation times for carbonyl carbons of triacylglycerol in different types of oils and fats[a]

		T_1[b] (s)	
Sample	Acyl chain	*sn*-1,3[c]	*sn*-2[c]
Standard triacylglycerols mixture	Saturated	3.93 ± 0.20	2.90 ± 0.10
	Monounsaturated	3.64 ± 0.30	2.84 ± 0.21
	Polyunsaturated	3.52 ± 0.04	2.64 ± 0.05
Palm olein IV56	Saturated	4.33 ± 0.12	2.96 ± 0.18
	Monounsaturated	3.97 ± 0.55	3.03 ± 0.15
	Polyunsaturated	4.62 ± 0.02	2.89 ± 0.35
Rapeseed oil (canola)	Saturated	4.32 ± 0.24	na
	Monounsaturated	3.67 ± 0.31	3.02 ± 0.16
	Polyunsaturated	4.03 ± 0.20	3.02 ± 0.09
Soybean oil	Saturated	4.11 ± 0.22	na
	Monounsaturated	3.90 ± 0.33	3.10 ± 0.17
	Polyunsaturated	4.62 ± 0.44	2.89 ± 0.09
Sunflower oil	Saturated	3.74 ± 0.33	na
	Monounsaturated	3.60 ± 0.45	2.74 ± 0.04
	Polyunsaturated	4.18 ± 0.32	3.38 ± 0.10
Grapeseed oil	Saturated	4.26 ± 0.42	na
	Monounsaturated	3.82 ± 0.30	3.10 ± 0.38
	Polyunsaturated	4.01 ± 0.48	3.32 ± 0.22
Rice bran oil	Saturated	3.62 ± 0.18	na
	Monounsaturated	3.60 ± 0.03	2.81 ± 0.19
	Polyunsaturated	3.96 ± 0.09	3.38 ± 0.18
Mango fat	Saturated	4.33 ± 0.26	na
	Monounsaturated	4.04 ± 0.10	2.74 ± 0.20
	Polyunsaturated	3.75 ± 0.11	2.60 ± 0.05
Olive oil	Saturated	3.90 ± 0.05	na
	Monounsaturated	3.82 ± 0.06	3.05 ± 0.17
	Polyunsaturated	4.33 ± 0.33	2.89 ± 0.11
Jatropha oil	Saturated	4.18 ± 0.02	na
	Monounsaturated	4.04 ± 0.51	2.86 ± 0.04
	Polyunsaturated	4.33 ± 0.29	3.18 ± 0.28

[a] na = not applicable due to absence of saturated acyl chain in *sn*-2 position. [b] Accuracies of T_1 are quoted as standard deviation of the mean of three replicates. ($n = 3$). [c] ANOVA showed there is significant difference in T_1 between *sn*-1,3 and *sn*-2 positions ($P < 0.01$).

triacylglycerols analyzed is found to be 4.62 seconds in palm olein at 9.4 Tesla (Table 3). Hence, 5.0 seconds is chosen as the longest T_1 for more general regiospecific analysis.

As shown in Fig. 1, in order to achieve 99% recovery of M_z after excitation by 90° pulse, the minimum repetition time must be at least 23.0 seconds (4.6 times of longest T_1). This figure will increase tremendously to 34.5 seconds (6.9 times of longest T_1) and 46.1 seconds (9.2 times of longest T_1) if targeted accuracies are set at 99.9% and 99.99% recovery of M_z, respectively. After acquisition time, the sample also needs adequate interval to achieve thermal equilibrium in order to prevent additional line broadening of resonances. Thus, sufficient pulse delay is required to minimize unfavorable sample heating effects due to proton decoupling during acquisition of FID in the inverse gated pulse sequence.[27,28] This also allows complete suppression of NOE which is evolved during acquisition due to proton decoupling. On the other hand, addition of paramagnetic relaxation reagent, namely, chromium(III) acetylacetonate, $Cr(acac)_3$, can dramatically reduce repetition time. Nonetheless, in the present study, the separation between monounsaturated and polyunsaturated carbonyl carbon resonances is diminished even at a low concentration of $Cr(acac)_3$. This will pose complications in analysis of fish oil samples. As a result, a repetition time of 34.5 s (5.4 s of acquisition time and 29.1 s of pulse delay) which is corresponding to 99.9% recovery, is recommended for general and practical regiospecific analysis of triacylglycerols by qCNMR at 9.4 Tesla.

3.1.3 Pulse angle selection. In quantitative NMR spectroscopy, 90° pulse is usually employed as it gives the maximum sensitivity for highest *S*/*N*. Nevertheless, long repetition time is required to allow the equilibrium magnetization state to be re-established before the application of subsequent pulse. In order to elucidate the optimum pulse angle for qCNMR regiospecific analysis, a comparison between 90° pulse and 30° pulse has been made.

As shown in Fig. 1, repetition delays required to achieve 99.9% recovery of M_z in equilibrium for 90° pulse and 30° pulse are 34.5 seconds and 24.5 seconds, respectively. For accumulation of 128 scans, the total experiment time will be 73.6 minutes during employment of 90° pulse, whereas the analysis time is reduced to 52.3 minutes for 30° pulse. There is significant time-saving for using a smaller pulse angle. However, such reduction is found to cause significant attenuation in the *S*/*N*. *S*/*N* ratios for the resultant spectrum acquired using 90° and 30° pulses are found to be 153 : 1 and 83 : 1, respectively. The

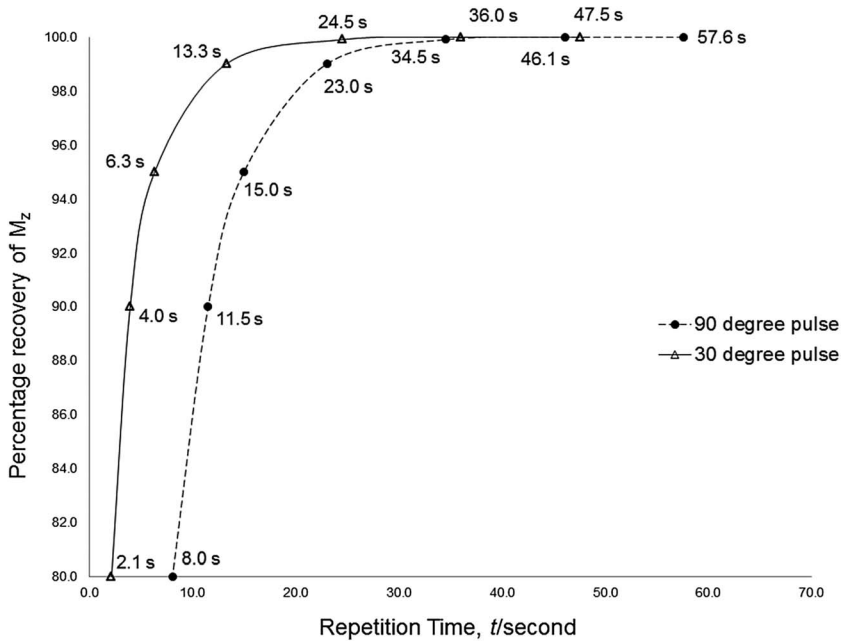

Fig. 1 Relationship between targeted accuracy and the minimum required repetition time, *t*. Triangles and circles correspond to experiments with 30° and 90° pulse angle, respectively. The longest T_1 of carbonyl carbons is set at 5.0 seconds.

equilibrium magnetization, M_o, and transverse magnetization, M_y, are related by

$$M_y = M_0 \sin\theta \exp^{-(t/T_2)} \quad (2)$$

T_2 is the spin–spin relaxation time. According to eqn (2), if the pulse angle is 30°, the M_y detected is only 0.5 times M_o, whereas for pulse angle of 90°, all of the initial M_o is converted into the detected M_y. Consequently, the *S*/*N* ratio with 90° pulse is twice that obtained with 30° pulse. Therefore, for experiments under employment of 30° pulse, the number of scans needs to be quadrupled since the increment of *S*/*N* is correlated by the square root of the number of signals taken. As a result, the total experiment time using 30° pulse will be 209.2 minutes, 2.8 times longer than that required by 90° pulse. In addition, other researchers also suggested the utilization of Ernst angle in quantitative NMR in an effort to reduce the experiment time.[27,28] Utilization of 90° flip angle is more time-saving as compared to smaller angles and eliminates problems due to Ernst angle. The latter, in fact, is maximizing steady-state signal intensities which strongly depend on the repetition rates and the individual relaxations, as well as different NOE of the carbon resonances. The effort in obtaining a fully relaxed magnetization during repetition time is not taken into consideration. As a result, Ernst angle, which is smaller than 90°, is favorable for structural elucidation, but not in qCNMR.

3.1.4 Temperature. Oil sample is generally viscous and a previous study suggested that high temperatures (50 °C) were necessary for achieving good resolution in the spectrum of carbonyl carbons.[22] As line width is inversely proportional to spin–spin relaxation time, T_2, narrower lines are expected at high temperature. Yet in our investigation, the differences in peak width and *S*/*N* ratio are not significant as the experimental temperature is being elevated, as shown in Fig. 2 by the *cis*-9 monounsaturated carbonyl carbon resonance of rapeseed oil (canola). When the experimental temperature is elevated from 20 °C to 50 °C, *S*/*N* ratio remains the same (153 : 1), whereas the smallest peak widths are found at 20 °C (0.31090 Hz) and 40 °C (0.31663 Hz). Nonetheless, the differences are not significant. In the event of measurement at elevated temperature, substantial waiting time is also required before shimming to achieve desired temperature and thermal equilibrium. Consequently, the experimental time is delayed. Moreover, the superconducting magnet is more difficult to shim at high

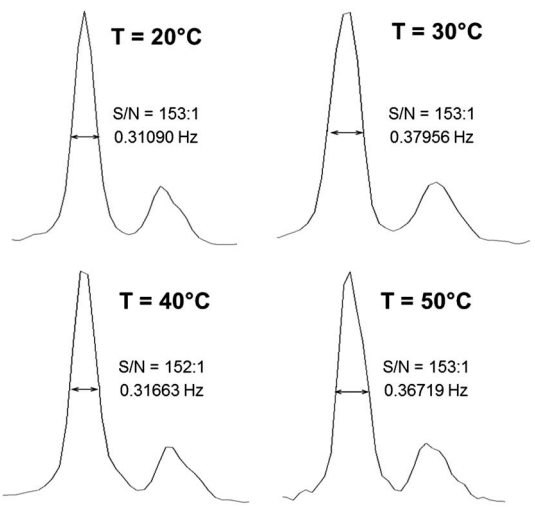

Fig. 2 Effect of experimental temperature (T = 20 °C, 30 °C, 40 °C and 50 °C) on peak width and *S*/*N* ratio of *cis*-9 monoene acyl chain carbonyl resonances of triacylglycerols in rapeseed oil (canola). Temperature control was used to maintain constant temperature throughout the experiment.

temperature, especially at temperatures near the boiling point of deuterated solvent. Therefore for routine measurement, the qCNMR regiospecific analysis at room temperature (25 °C), with the aid of temperature control, is recommended.

3.2 Data processing parameters

Careful manual phasing of the spectrum ensures that the integrals representing the carbonyl carbon resonances will have minimal distortion. In addition, the baseline correction must be applied prior to deconvolution as it will further assure the quantitative accuracy and reproducibility.

3.2.1 Zero filling. In qNMR, zero filling was reported to have no effect on precision of peak integration.[29] Yet in another study of systematic errors due to integration of NMR spectra, one level of zero filling was found to be sufficient for reducing the systematic integration error, providing that a 1% integration limit was used.[30] On the contrary, zero filling was suggested to be omitted in order to ensure unbiased quantitative information obtained from experimental data.[31] Due to aforementioned controversial disclosures in prior literature, concrete justification is done in practical regiospecific analysis by qCNMR. In the analysis of synthetic mixture of standard triacylglycerols, as shown in Table 4, one level of zero filling is found to yield low repeatability and precision, as indicated by the wide-ranging standard deviation (0.8–2.6 mol%), as compared to that when no zero filling is used (0.3–0.8 mol %). All the data points of the real and imaginary part of the spectrum should be uncorrelated, as long as zero filling is omitted. Although the accuracies are not affected, this artificial data processing has subjected the quantitative results to a greater extent of systematic errors and unrepeatability. On the condition that the data points are sufficient for FID to fully decay, zero filling should be omitted.

3.2.2 Apodization. In current qCNMR regiospecific analysis, the FID is allowed to decay within 5.4 seconds. Since all FID are supposed to decay exponentially with time, the exponential tends to be the most suitable window function in data processing. In an effort to weight the noise-rich tail of FID towards zero, different levels of line broadening can be employed, but at the cost of peak resolution. Table 5 shows the errors in results of the synthetic mixture of standard triacylglycerols, which are caused by different extents of line broadening. The standard deviations of quantitative results after excessive line broadening (0.1–0.4 Hz) fall in the range of 0.7–4.8 mol%. Apart from depreciation in precision, accuracy also drops tremendously if higher extent of line broadening is employed. This is exhibited by the composition of mono- and polyunsaturated carbonyl carbon resonances, as compared to the actual values of the synthetic mixture (Table 5). Under 0.2 Hz and 0.4 Hz factors of line broadening, the mono- and polyunsaturated carbonyl carbon resonances are poorly resolved, which subsequently cause deviated quantitative results. The greatest deviation (11.1 mol%) is found for the polyunsaturated carbonyl carbon resonance at *sn*-2 position, under line broadening 0.4 Hz. As a result, for unbiased qCNMR regiospecific analysis, a minimal line broadening factor of 0.01 Hz is highly recommended, whereas the excessive artificial data processing methods which aim to improve *S*/*N* must be avoided. Furthermore, the peak widths for all resonances of interest must be kept constant throughout the deconvolution or integration process.

3.3 Validation on the oils and fats from different sources

The current qCNMR regiospecific analysis has been successfully employed as a monitoring tool in sodium methoxide-catalyzed chemical interesterification of palm olein. The compositions at both *sn*-1,3 and *sn*-2 are completely randomized and resemble the total fatty composition, as shown in Table 6 at duration of 30 minutes, affirming that the interesterification has reached the equilibrium state.

On the other hand, triacylglycerol content extracted from visceral fat of C57BL/6 mice after 15 weeks feeding with palm

Table 4 Study of the effect of zero filling on the accuracy and precision of qCNMR regiospecific analysis of standard triacylglycerols

	Composition[a] (mol%)		
Carbonyl carbon signals	Without zero filling	After one level of zero filling	Actual (by synthetic mixing)
***sn*-1,3**			
Saturated	26.2 ± 0.8	27.0 ± 1.3	26.7
Monounsaturated	61.1 ± 0.3	59.3 ± 2.6	60.6
Polyunsaturated	12.7 ± 0.8	13.7 ± 1.8	12.7
***sn*-2**			
Saturated	25.5 ± 0.2	27.0 ± 1.0	26.7
Monounsaturated	60.2 ± 0.5	60.0 ± 1.4	60.6
Polyunsaturated	14.3 ± 0.7	13.0 ± 0.8	12.7
Overall			
Saturated	26.0 ± 0.6	27.0 ± 1.1	26.7
Monounsaturated	60.8 ± 0.3	59.5 ± 2.2	60.6
Polyunsaturated	13.2 ± 0.8	13.5 ± 1.4	12.7

[a] Values are the mean of five replicates ± standard deviation on the same FID.

Table 5 Study of the line broadening (LB) effect on the accuracy and precision of qCNMR regiospecific analysis of standard triacylglycerols

	Composition[a] (mol%)				
Carbonyl carbon signal	LB factor[b] 0.01 Hz	LB factor[b] 0.1 Hz	LB factor[b] 0.2 Hz	LB factor[b] 0.4 Hz	Actual (by synthetic mixing)
***sn*-1,3**					
Saturated	26.2 ± 0.8	25.7 ± 3.5	26.7 ± 3.4	26.4 ± 3.0	26.7
Monounsaturated	61.1 ± 0.3	62.4 ± 2.6	59.5 ± 3.3	53.2 ± 3.1	60.6
Polyunsaturated	12.7 ± 0.8	11.9 ± 1.5	13.8 ± 1.1	20.4 ± 2.5	12.7
***sn*-2**					
Saturated	25.5 ± 0.2	29.5 ± 2.1	28.4 ± 0.7	26.1 ± 2.0	26.7
Monounsaturated	60.2 ± 0.5	60.0 ± 1.5	54.5 ± 2.9	50.2 ± 3.0	60.6
Polyunsaturated	14.3 ± 0.7	10.5 ± 0.8	17.1 ± 2.5	23.8 ± 4.8	12.7
Overall					
Saturated	26.0 ± 0.6	26.9 ± 2.2	27.1 ± 2.4	26.3 ± 2.7	26.7
Monounsaturated	60.8 ± 0.3	61.6 ± 1.8	58.0 ± 2.6	52.2 ± 2.2	60.6
Polyunsaturated	13.2 ± 0.8	11.5 ± 0.9	14.8 ± 1.4	21.5 ± 2.9	12.7

[a] Values are mean of five replicates ± standard deviation on the same FID. [b] All FIDs are processed in exponential window function.

Table 6 Wide application of qCNMR regiospecific analysis using established parameters[a]

		Composition[b] (mol%)			
			Monounsaturated		
Sample	*sn*-position	Saturated	*Cis*-11	*Cis*-9	Polyunsaturated
Palm olein IV 56	1, 3	71.0 ± 0.4	nd	26.1 ± 0.9	2.9 ± 0.9
	2	7.4 ± 0.9	nd	74.3 ± 0.9	18.3 ± 0.9
	1, 2, 3	50.3 ± 0.4	nd	41.8 ± 0.9	7.9 ± 0.9
Chemical interesterified palm olein ($t = 8$ minutes)	1, 3	61.3 ± 0.3	nd	31.7 ± 0.3	6.9 ± 0.0
	2	22.1 ± 0.7	nd	59.9 ± 0.4	18.0 ± 0.3
	1, 2, 3	48.3 ± 0.2	nd	41.1 ± 0.2	10.6 ± 0.1
Chemical interesterified palm olein ($t =$ 30 minutes)	1, 3	49.6 ± 0.6	nd	39.9 ± 0.8	10.5 ± 0.6
	2	49.0 ± 0.8	nd	41.0 ± 0.7	10.0 ± 0.7
	1, 2, 3	49.2 ± 0.8	nd	40.5 ± 0.8	10.3 ± 0.6
Visceral fat from mice fed with palm olein	1, 3	35.7 ± 0.6	7.6 ± 0.4	46.1 ± 0.6	10.6 ± 0.8
	2	9.0 ± 0.8	nd	62.0 ± 0.6	29.0 ± 0.9
	1, 2, 3	26.6 ± 0.7	5.0 ± 0.5	51.5 ± 0.6	16.9 ± 0.9
Soybean oil	1, 3	26.3 ± 0.6	2.0 ± 0.4	23.4 ± 0.9	48.3 ± 0.2
	2	nd	nd	23.8 ± 0.5	76.2 ± 0.5
	1, 2, 3	17.5 ± 0.4	1.3 ± 0.3	23.5 ± 0.6	57.7 ± 0.1
Visceral fat from mice fed with soybean oil	1, 3	22.6 ± 0.5	8.2 ± 0.4	31.6 ± 0.8	37.6 ± 0.9
	2	nd	nd	33.0 ± 0.4	67.1 ± 1.1
	1, 2, 3	14.9 ± 0.4	5.4 ± 0.4	32.1 ± 0.6	47.6 ± 1.0
Jatropha oil	1, 3	36.3 ± 0.7	nd	38.6 ± 1.8	25.2 ± 1.8
	2	nd	nd	44.6 ± 1.3	55.5 ± 1.3
	1, 2, 3	24.9 ± 0.7	nd	40.5 ± 1.9	34.6 ± 1.6
Mango olein	1, 3	48.7 ± 0.9	nd	45.5 ± 0.4	5.8 ± 0.7
	2	nd	nd	82.1 ± 0.9	17.8 ± 0.9
	1, 2, 3	32.8 ± 0.9	nd	57.8 ± 0.7	9.4 ± 0.4
Shea butter	1, 3	51.8 ± 1.7	nd	37.7 ± 1.7	10.5 ± 0.1
	2	nd	nd	73.2 ± 1.4	26.8 ± 1.4
	1, 2, 3	33.9 ± 1.5	nd	49.9 ± 1.3	16.1 ± 0.5
Cocoa butter	1, 3	95.0 ± 0.8	nd	5.0 ± 0.7	nd
	2	7.8 ± 0.6	nd	92.2 ± 0.5	nd
	1, 2, 3	65.4 ± 0.7	nd	34.6 ± 0.7	nd
Sal stearin	1,3	93.7 ± 0.2	nd	4.6 ± 0.7	1.7 ± 0.7
	2	nd	nd	100.0 ± 0.0	nd
	1, 2, 3	62.4 ± 0.2	nd	36.5 ± 0.4	1.1 ± 0.7

[a] nd = not detectable. [b] ^{13}C NMR results are mean of three replicates ± standard deviation.

olein IV 56 shows the content of saturated fatty acids at *sn*-1,3 positions decreasing dramatically from 71.0 mol% in the diet, to 35.7 mol% after deposited at visceral cavity (Table 6). A slight decrement (3.7 mol%) is also observed in visceral fat of mice fed with soybean oil. This is correlated to the poorly absorbed long chain saturated fatty acids in the intestine by virtue of the formation of calcium soaps, and subsequently subjected to fecal excretion.[32] In addition, 7.6 mol% of *cis*-11 monounsaturated fatty acid is detected at *sn*-1,3 positions of triacylglycerols in visceral fat, which is absent in the fed palm olein (Table 6). An increment of 6.2 mol% of similar fatty acid is also observed in visceral fat of mice fed with soybean oil, suggesting the occurrence of *cis*-vaccenic acid in adipose tissues.[33] *Cis*-vaccenic acid (C18:1 *n*-7) can be easily distinguished from oleic acid (C18:1 *n*-9) and further determined by its positional distribution using current qCNMR method.

The current method has been applied to determine the positional fatty acid compositions in selected oils and fats, ranging from relatively liquefied *Jatropha* oil and mango olein, to highly solidified shea, cocoa butter and sal stearin, without their regiospecific data reported up-to-date. On the other hand, the application on menhaden fish oil is demonstrated in Fig. 3 and the corresponding positional fatty acid compositions are tabulated in Table 7. The signals of carbonyl resonance are in agreement with previous work.[9,17,18,23] Despite the presence of more complex polyunsaturated fatty acids, the peak assignment follows the trend as explained earlier. Carbonyl carbons of acyl groups from *sn*-1,3 positions appear at higher frequencies than those residing at the *sn*-2 position. However, docohexaenoic acid (DHA) exhibits remarkable chemical shift compared with the other fatty acids, as the difference in chemical shift from saturated species was found greatest if first double bond occurred at γ-position from carbonyl carbons.[34]

Table 7 qCNMR regiospecific analysis on menhaden fish oil[a]

	Composition[c] (mol%)		
Acyl chain[b]	*sn*-1,3	*sn*-2	Total
Saturated	42.9 ± 1.9	32.7 ± 0.6	38.2 ± 0.9
Monounsaturated, Δ9	21.8 ± 0.7	11.3 ± 0.8	16.9 ± 0.1
Polyunsaturated, Δ9	nd	2.6 ± 0.4	1.2 ± 0.2
C20:4, Δ8	3.5 ± 0.2	1.3 ± 0.2	2.5 ± 0.1
C22:5, Δ7	2.3 ± 0.1	4.0 ± 0.4	3.1 ± 0.2
C18:4, Δ6	5.2 ± 0.2	6.7 ± 0.6	5.9 ± 0.2
C20:5, Δ5	15.6 ± 0.5	15.7 ± 0.3	15.6 ± 0.4
C22:6, Δ4	8.7 ± 0.3	25.7 ± 0.8	16.6 ± 0.8

[a] nd = not detectable. [b] The acyl chains are presented according to the sequence of their resonance from higher to lower frequency. [c] ^{13}C NMR results are the mean of three replicates ± standard deviation.

Within similar *sn*-position, the peak assignment follows the distance of the first double bond from the carbonyl carbon, *viz.*, the resonances are shifted to lower frequency from Δ9-fatty acid to Δ5-fatty acid. Due to the high electron density of the double bond, it tends to exert shielding effect on the electron-withdrawing carbonyl group, causing the carbon resonance to appear at a lower frequency. It is interesting to note that the chemical shift is highly influenced by the position of the first double bond from carbonyl carbon, instead of the total number of double bonds in the fatty acid chain. The presence of unsaturation beyond two double bonds, within the identical position of first double bond, could not be distinguishable in the region of carbonyl carbon resonances.[34] We herewith present the first approach with the employment of a narrower spectral width (1500 Hz at which the carbonyl carbon resonates) in qCNMR regiospecific analysis on fish oil, targeting to provide full quantitative data in shorter analysis. With the set of acquisition

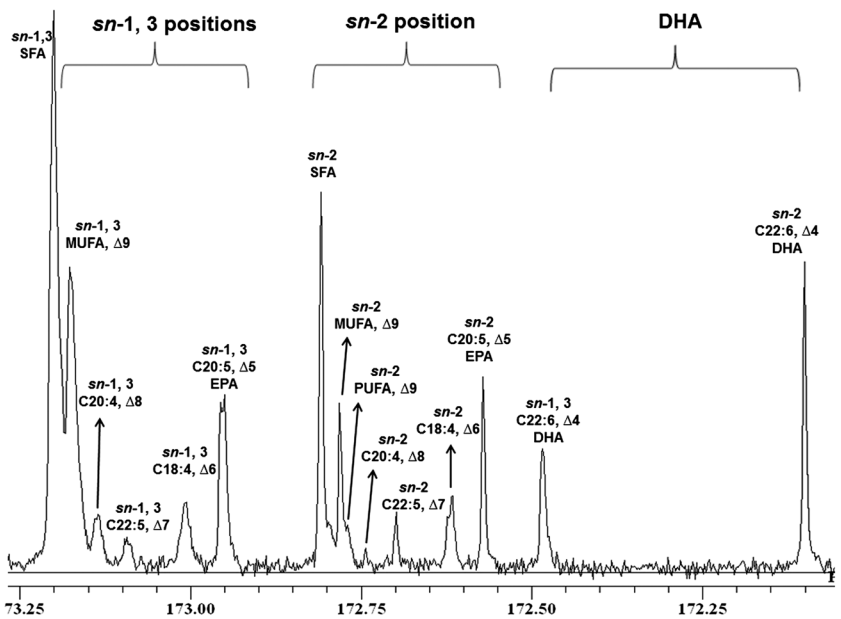

Fig. 3 400 MHz ^{13}C NMR spectrum of acyl chain carbonyl resonances (spectral width = 1500 Hz) of triacylglycerols in menhaden fish oil: SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, EPA eicosapentaenoic acid, DHA docosahexaenoic acid.

parameters and appropriate FID data processing method established, error of less than 2.0 mol% can be achieved coupled with high repeatability and versatility on the fast analysis of oils and fats from diverse sources.

4 Conclusions

Current selection of data acquisition and processing parameters in qCNMR enable us to establish a more elegant, rapid, accurate and unbiased regiospecific analysis of triacylglycerols from oils and fats which can be generalized and be used as a cook book. We also have shown that inappropriate NMR data acquisition and processing methods reported previously can render bias quantitative results. Apart from application in regiospecific analysis, the current methodology developed can be extended as a fast reaction monitoring tool, particularly for interpretation of the rate of enzymatic acidolysis, transesterification and interesterification by analyzing the positional distribution of fatty acids in the intermediate reaction products. Moreover, the present considerations are useful for other qCNMR experiments, for instance in the quantitative analysis of olefinic carbons in triacylglycerols or other types of compounds, and even can be further extended to other types of nuclei, namely quantitative ^{15}N, ^{29}Si and ^{31}P NMR spectroscopy.

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